



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C08B 37/10, A61K 31/725		A1	(11) International Publication Number: WO 92/01003
			(43) International Publication Date: 23 January 1992 (23.01.92)
(21) International Application Number: PCT/US91/04863		(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(22) International Filing Date: 10 July 1991 (10.07.91)			
(30) Priority data: 550,827 10 July 1990 (10.07.90) US			
(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).			
(72) Inventors: NICOLSON, Garth, L. ; 2611 Valley Manor, Kingwood, TX 77339 (US). IRIMURA, Tatsuro ; 5205 Evergreen, Bellaire, TX 77401 (US). NAKAJIMA, Moto-wo ; 5803 Dryad, Houston, TX 77035 (US).			
(74) Agent: HODGINS, Daniel, S.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).			

(54) Title: GLYCOSAMINOGLYCAN DERIVATIVES AND THEIR USE AS INHIBITORS OF TUMOR INVASIVENESS OR METASTATIC PROFUSION-II

(57) Abstract

The present invention comprises a method for impeding the formation of tumor metastasis or tumor invasiveness in a host. Such inhibition comprises administration to the host of a glycosaminoglycan derivative substantially devoid of anticoagulation activity and is an effective inhibitor of heparanase activity. Such a glycosaminoglycan derivative may be provided by purchase or synthesis as directed herein. Parenteral administration to a tumor-bearing host of the glycosaminoglycan derivative results in the exposure of host-borne tumor cells thereto. Such exposure to effective levels of the derivative results in the inhibition of tumor heparanase activity and a lessening of invasiveness and metastatic spread. Heparin, a glycosaminoglycan particularly effective as a heparanase inhibitor and an anti-clotting agent, is a preferred glycosaminoglycan for derivatization. Upon derivatization according to the present invention heparin may be converted into a glycosaminoglycan derivative substantially devoid of anticoagulant activity but yet being an effective inhibitor of heparanase activity. Mere reduction of heparin carboxyl groups results in the production of a glycosaminoglycan derivative inhibitory to heparanase activity but without substantially anticoagulant activity. non-anticoagulating, heparanase-inhibiting glycosaminoglycan derivatives may also be prepared from heparin, for example, by: at least partial N-desulfation and then N-acetylation; or N-, O-desulfation followed by N-resulfation.

+ DESIGNATIONS OF "SU"

It is under examination in which parts of the former Soviet Union the designation of the Soviet Union has effect.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

5

10

**GLYCOSAMINOGLYCAN DERIVATIVES AND THEIR
USE AS INHIBITORS OF TUMOR INVASIVENESS
OR METASTATIC PROFUSION-II**

15

The present invention relates to glycosaminoglycan derivatives useful in the inhibition of endoglycosidase activity and of tumor invasiveness or metastatic spread.

20

A class of biological substances called the proteoglycans form the ground substance in the extracellular matrix of connective tissues. These proteoglycans are polyanionic substances of high molecular weight and contain many different types of heteropolysaccharide side chains covalently linked to a polypeptide backbone. These proteoglycans may contain over 95% carbohydrates. The polysaccharide groups of the proteoglycans were formerly called mucopolysaccharides but now are preferably termed glycosaminoglycans since all contain derivatives of glucosamine or galactosamine.

30

A variety of enzymes may be involved in the normal metabolic degradation of proteoglycans. Initial proteoglycan degradation often involves proteolysis to separate or digest protein components. Such proteolysis results in the production of glycosaminoglycans. The glycosaminoglycans in turn are subject to glycosaminoglycan endoglycosidase enzymic action which produces smaller glycosaminoglycan fragments. The glycosaminoglycans or fragments thereof are subject to glycosaminoglycan exoglycosidase enzymic action which produces

35

40

monosaccharides from the non-reducing ends of glycosaminoglycans.

5 An increasing interest in the endoglycosidases has arisen in recent years because of a possible relationship of these enzymes with tumor invasiveness and tumor meta- static activity. Nicolson (1982, Biochem. Biophys. Acta. V 695, pp 113-176) reviewed a variety of oligosaccharide-
10 degrading enzymes (pp 141-142) reported to be of interest in malignant disease. Nicolson (1982, J. Histochem. & Cytochem. V 30, pp 214-220) described a proposed mechanism for tumor cell invasion of endothelial cell basal lamina and a related production of degradation
15 products from proteins and glycosaminoglycans. Kramer et al., (1982, J. Biol. Chem. V 257, pp 2678-2686) reported a tumor-derived glycosidase capable of cleaving specifically glycosaminoglycans and releasing heparan sulfate-rich fragments.

20 Irimura et al., (1983a, Analyt. Biochem. V 130, pp 461-468) describe high-speed gel-permeation chromatography of glycosaminoglycans. Heparan sulfate degrading activity of melanoma cells was measured by using this chromatographic procedure. Nakajima et al.,
25 (1983, Science, V 220, pp 611-613) described a relationship of metastatic activity and heparan sulfate degrading activity in melanoma cell lines. The disappearance of higher molecular weight heparan sulfate was followed by polyacrylamide gel electrophoresis,
30 staining and densitometry.

Vlodavsky et al., (1983, Cancer Res. V 43, pp 2704-2711) described the degradation by two T-lymphoma cell lines of ³⁵S labeled proteoglycans from confluent endo-
35 thelial cells. The highly metastatic line had much

higher ^{35}S liberating activity than did the low metastatic line.

Irimura et al., (1983c, Proc. Am. Soc. Cancer Res. V
5 24, p 37, abstract 144), using high performance liquid
chromatography, describe heparan sulfate degradative
enzyme activity of melanoma cells. Nakajima et al.,
(1984, J. Biol. Chem. V 259, pp 2283-2290) describe
characterizations of metastatic melanoma heparanase.
10 High speed gel permeation chromatography and chemical
analyses were used in a description of functional
substrates and products formed. Nakajima et al. (1986,
Anal. Biochem., in press) synthesized a solid-phase
substrate for heparanase by crosslinking radiolabeled and
15 reductively aminated HS to amino-reactive agarose beads
via one covalent linkage. This solid-phase substrate was
used for the measurement of heparanase activity in
various human melanoma cell lines (Nakajima et al.,
(1986) Cancer Letters, V 31, pp 277-283) and sera from
20 mammary adenocarcinoma-bearing rats and malignant
melanoma patients (Nakajima et al., (1986) In: Cancer
Metastasis : Experimental and Clinical Strategies, D.R.
Welch, B.K. Bhuyan, L.A. Liotta, eds. Alan R. Liss, Inc.,
New York, pp 113-122).

25 From the foregoing it may be seen that significant
interest exists in convenient, accurate and reproducible
endoglycosidase assays and production of potent
heparanase inhibitors, particularly since endogly-
30 cosidases may play critical roles in the establishment of
tumor metastases.

The ability of tumor cells to invade host tissues
and metastasize to distant, often specific organ sites,
35 is one of their most important properties. Metastasis
formation occurs via a complex series of unique

interactions between tumor cells and normal host tissues and cells. These processes involve several discrete and selective steps such as: invasion of surrounding tissues, penetration of lymphatics of blood vessels and transport in lymph or blood, or dissemination into a serous cavity, arrest and invasion at distant sites, and survival and growth to form secondary lesions.

Basement membranes are continuous sheets of extra-cellular matrix composed of collagenous and non-collagenous proteins and proteoglycans that separate parenchymal cells from underlying interstitial connective tissue. They have characteristic permeabilities and play a role in maintaining tissue architecture. Metastasizing tumor cells must penetrate epithelial and endothelial basement membranes during invasion and metastasis, and the penetration and destruction of basement membranes by invasive tumor cells has been observed using electron microscopy. Since basement membranes are rigid structures formed from unique sets of macromolecules, including type IV collagen, laminin, heparan sulfate (HS), proteoglycan and fibronectin, the successful penetration of a basement membrane barrier probably requires the active participation of more than one tumor cell-associated enzyme.

Due to its unique physical and chemical properties such as its polyanionic character and barrier properties against macromolecules (Kanwar et al., 1980 J. Cell. Biol. V 86, pp 688-693), HS is an important structural component of basement membranes. HS binds to fibronectin, laminin and type IV collagen, and these molecules have been collectively observed in the basal lamina using antibodies raised against each component. HS may be involved in basal lamina matrix assembly by promoting the interactions of collagenous and non-

-5-

collagenous protein components while protecting them against proteolytic attack. Thus, the destruction of HS proteoglycan barrier could be important in basement membrane invasion by tumor cells.

5

The interactions between malignant cells and vascular endothelium have been studied using monolayers of cultured vascular endothelial cells that synthesize an extracellular matrix resembling a basement membrane. With this model, it has been found that metastatic B16 melanoma cells degrade matrix glycoproteins, such as fibronectin, and matrix sulfated glycosaminoglycans, such as heparan sulfate. Since HS was released in solution as fragments approximately one-third their original size, it has been proposed that metastatic tumor cells characteristically have a HS endoglycosidase.

The relation between metastatic properties and the ability of five B16 melanoma sublines of various implantation and invasion characteristics to enzymatically degrade subendothelial extracellular matrix indicated that highly invasive and metastatic B16 sublines degraded sulfated glycosaminoglycans faster than did sublines of lower metastatic potential (Nakajima et al., (1983), Science V 220, p 611), and intact B16 cells (or their cell-free homogenates) with a high potential for lung colonization also degraded purified heparan sulfate at higher rates than did B16 cells with a poor potential for lung colonization (ibid).

30

The abilities of B16 cells to degrade HS from various origins and other purified glycosaminoglycans (heparin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid) have been studied. In order to analyze glycosaminoglycan degradation products, an analytic procedure was developed

35

using high-speed gel permeation chromatography (Irimura et al., (1983a) Anal. Biochem. v 130, p 161; Nakajima et al., (1984) J. Biol. Chem. v 259, p 2283). HS metabolically labeled with ^{35}S -sulfate was purified from basement membrane producing EHS sarcoma and PYS-2 carcinoma cells, and subendothelial matrices of bovine aortic endothelial (BAE) and corneal endothelial (BCE) cells (ibid). HS molecules purified from bovine lung and other glycosaminoglycans were labeled with tritium at their reducing termini using ^3H - NaBH_4 . These labeled glycosaminoglycans were incubated with B16 cell extracts in the absence or presence of D-saccharic acid 1,4-lactone, a potent exo-beta-glucuronidase inhibitor, and degradation fragments were analyzed by high-speed gel permeation chromatography.

HS isolated from the various origins described above were all degraded into fragments of characteristic molecular weight, in contrast to hyaluronic acid, chondroitin 6-sulfate, chondroitin 4-sulfate, dermatan sulfate, keratan sulfate, and heparin, which were essentially undegraded. Heparin, but not other glycosaminoglycans, inhibited HS degradation. The time dependence of HS degradation into particular molecular weight fragments indicated that melanoma heparanase cleaves HS at specific intrachain sites (ibid). In order to determine specific HS cleavage points, the newly formed reducing termini of HS fragments were investigated by: labeling with ^3H - NaBH_4 ; hydrolysis to monosaccharides; and analysis of these saccharides by paper chromatography. Since ^3H -reduced terminal monosaccharides from HS fragments were overwhelmingly (>90%) L-gulonic acid, the HS-degrading enzyme responsible was an endoglucuronidase (heparanase).

HS-degrading endoglucuronidases have been found in various tissues, such as human skin fibroblasts, rat

-7-

liver cells, human placenta, and human platelets. HS-degrading endoglucuronidases in mammalian cells were reported previously by other investigators to be "heparitinases" to indicate heparitin sulfate (heparan sulfate)-specific endoglycosidase. However, heparitinase originally was used to designate an elimination enzyme (EC 4.2.2.8) in Flavobacterium heparinum, and this enzyme cleaves nonsulfate and monosulfated 2-acetoamido-2-deoxy-alpha-D-glucosyl-D-hexuronic acid linkages of HS. Since HS-specific endoglycosidases in mammalian cells are endoglucuronidases, except for one found in skin fibroblasts, it was proposed that mammalian cell endoglucuronidases capable of degrading HS should be called "heparanases", consistent with the currently used term "heparan sulfate".

High heparanase activity in human melanoma cells was demonstrated using a solid-phase substrate, partially N-desulfated N-[¹⁴C] acetylated HS crosslinked to agarose beads via one covalent linkage (Nakajima et al., (1986) Cancer Letters, V 31, pp 277-283; Nakajima et al., (1986) Anal. Biochem. in press). All of the 15 human melanoma cell lines tested were found to have heparanase activity and almost all possessed high activities comparable or greater than that of the murine B16-F1 melanoma line. Human A375 melanoma variants of high lung metastatic potential in athymic nude mice had significantly higher heparanase activities than did A375 parental cells of low metastatic potential.

High heparanase activity was also found in the sera from highly metastatic tumor-bearing animals and malignant melanoma patients (Nakajima et al, (1986) In: Cancer Metastasis : Experimental and Clinical Strategies, D.R. Welch, B.K. Bhuyan, L.A. Liotta, eds., Alan R. Liss, Inc., New York, pp 113-122). A significant difference in

serum heparanase activity was found between a group of normal adults and a group of malignant melanoma patients (N=35, $p < 0.05$). The mean values of serum heparanase activities in normal adults and malignant melanoma patients were 3.97 and 9.43 mg HS/h/ml serum, respectively. Some of the patients having documented lymph node metastases had 4 to 6-fold higher serum heparanase activities than normal adults.

Anticoagulants, such as heparin, warfarin, dextran sulfate and Ancrod; (Agkistrodon rhodostoma venom protease, Abbott) have been used to prevent blood coagulation and reduce the formation of metastatic tumor cell thrombi in the blood which lodge more effectively in the microcirculation Hilgard *et al.*, Eur. J. Cancer, V 12, pp 755-762, 1976). H. Ludwig (Gynakologe, V 7, pp 1-10, 1974) reported longer recurrence-free intervals and less metastases in patients during irradiation of their gynaecological cancers. Heparin has been used as an adjuvant therapy agent with combination chemotherapy of inoperable lung cancer with enhanced therapeutic effects (Elias, Proc. Amer. Assoc. Cancer Res., V 14, p 26, 1973, Abst.). L. Michaels (Lancet, V 2, pp 832-83, 1964) found that the cancer incidence and death rate was lower than expected for patients receiving heparin. Dextran sulfate ($M_r - 7,000$) has been used to inhibit metastasis of rat lung tumors, and human lung cancer patients have received long term oral dextran sulfate. Although the anti-metastatic effects of dextran sulfate were marginal in cancer patients, this was probably due to the low intestinal absorption of dextran sulfate (Suemasu, Gann Monogr., V 20, pp 163-172, 1977).

Heparin and related sulfated glycoconjugates with anticoagulation properties, such as dextran sulfate have been used experimentally as antimetastatic agents

(Tsubura et al., (1977) Gann Monogr. Cancer Res., V 20, pp 147-153; Hilgard, (1984) in Cancer Invasion and Metastasis, Biologic and Therapeutic Aspects, Nicolson et al., eds, pp 353-360 Raven Press, N.Y.) The basis for
5 this use was the assumption that platelet aggregation, together with activation of the coagulation cascade, enhanced the formation of tumor embolism and increased implantation and metastatic colonization of blood-borne
10 tumor cells. In other studies on the effects of heparin on metastasis, however, heparin administration increased, decreased, or had no effect on tumor cell dissemination and organ colonization, depending on the experimental system. Mechanisms other than the anticoagulation
15 effects of heparin on tumor metastasis were suggested by these results, but the possible involvement of tumor heparanase had not been considered. The present invention relates to heparin derivatives without anti-coagulant properties and which inhibit the heparanase activity of metastatic mouse melanoma cells. These
20 substances were useful as tools for in vitro and in vivo studies involving the role of heparanase in tumor invasion and metastasis.

Using oral administration of heparin in combination
25 with hydrocortisone, it was reported that complete regression of established transplantable tumors in mice could occur through inhibition of tumor angiogenesis (Folkman et al., Science, V 221, pp 719-725, 1983). This suggested that anti-angiogenic substances could be used
30 for cancer therapy (J. Folkman in: Important Advances in Oncology, de Vita et al., eds, pp 42-62, J.B. Lippincott, Philadelphia, 1985). In such studies heparin was administered in the drinking water of animals. For
35 example, hamsters have been inoculated with transplantable pancreatic carcinoma cells and have been treated by receiving heparin or hexuronyl hexaminoglycan

sulfate, a heparin derivative, in their drinking water at concentrations of 10 mg/ml with hydrocortisone (0.5 mg/ml). After treatment for 6-9 days, the tumors were examined. In 3 out of 4 of the tumors the hexuronyl
5 hexaminoglycan sulfate plus hydrocortisone resulted in significant reductions in growth rate in vivo and significant inhibitions of capillary endothelial cell migration in Boyden chambers in vitro (Rong et al., Cancer, V 57, pp 586-590, 1986).

10

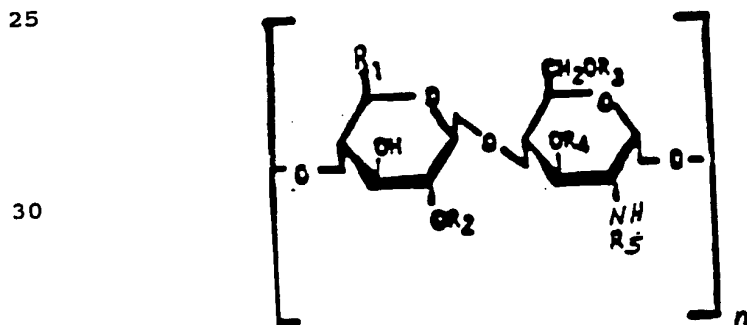
Proliferation of vascular smooth muscle cells has been shown to be an important step in the pathogenesis of arteriosclerosis (Ross et al., Science, V 180, pp 1332-1339, 1973). Commercial heparin preparations have been
15 separated into anticoagulant and non-anticoagulant mixtures by use of antithrombin to remove the anti-coagulant heparin. Both of these forms of heparin significantly inhibit the growth of smooth muscle cells in vitro (Hoover et al., Circulation Res., V 47, pp 578-583,
20 1983). Using the heparin preparations at a concentration of 10 ug/ml resulted in approximately 50% inhibition of ³H-thymidine uptake by arterial smooth muscle cells, which is indicative of growth inhibition. Administration of anticoagulating and non-anticoagulating heparin
25 fractions inhibited intimal smooth muscle proliferation, as determined by the total plaque volume two weeks after arterial injury. Non-anticoagulating heparin given at a dose of 100 USP units per kg body weight per hour in Sprague-Dawley rats resulted in 77% inhibition of
30 myointimal growth (Guyton et al., Circulation Res., V 46, pp 625-634, 1980).

Further studies indicated that the minimum fragment size of heparin, which was growth inhibitory toward
35 vascular smooth muscle cells, was a hexasaccharide, and the maximum anti-proliferative activity was obtained with

-11-

a 12-residue heparin. Most modified heparins (totally desulfated, N-desulfated, and totally desulfated re-N-acetylated) lost their anti-proliferative activity, but the N-desulfated N-resulfated heparin and the N-desulfated N-acetylated heparin retained full growth inhibitory properties (Castellot et al., J. Cell. Physiol., V 120, pp 315-320, 1984). Diseases, such as arteriosclerosis, develop over a long period of time. Therefore, the main use of such treatments might be in vascular damage due to trauma or surgery, such as artery vein grafts or arterio-venous shunts for kidney dialysis.

The present invention comprises novel compositions and a method for impeding the formation of tumor metastasis or tumor invasiveness in a host. The spread of melanomas and mammary carcinomas is inhibited by the derivatives described herein. The method comprises parenteral administration to the host of a glycosaminoglycan derivative substantially devoid of anticoagulation activity and which is an effective inhibitor of heparanase activity. Suitable glycosaminoglycan derivatives, which are useful as novel compositions in the method of the invention, include glycosaminoglycan derivatives of the following structure:



Where

35

R_1 is $-COOH$ or $-CH_2OH$ and the

-12-

configuration of the carbon atom to which R_1 is bound is D or L ;

R_2 is $-H$ or $-SO_3^-$

5

R_3 is $-H$ or $-SO_3^-$;

R_4 is $-H$ or $-SO_3^-$;

10

R_5 is $-H$, $-SO_3^-$ or $-CO-CH_3$;

n is 3 to 30; and

15

each of the terminal monomeric units is a monomeric repeating unit with the terminal oxygen atom being bound to a blocking group.

The blocking group may be a small alkyl or acyl group having less than about 5 carbon atoms and preferably is $-H$, $-CH_3$, $-SO_3$ or $CO-CH_3$.

The glycosaminoglycan derivatives of the present invention may be prepared by derivatization of heparin obtained from natural sources or they may be prepared by a variety of conventional synthetic techniques. Administration to a tumor-bearing host of an effective amount of a glycosaminoglycan derivative of the invention results in the exposure of host-borne tumor cells thereto. Such exposure to effective levels of the derivative results in the inhibition of tumor heparanase activity and a lessening of tumor invasiveness and metastatic spread.

Heparin, a glycosaminoglycan which is effective both as a heparanase inhibitor and an anti-clotting agent, is

-13-

a preferred glycosaminoglycan for derivatization. Upon derivatization according to the present invention, heparin may be converted into a glycosaminoglycan derivative substantially devoid of anticoagulant activity but yet being an effective inhibitor of heparanase activity. Reduction of heparin carboxyl groups results in the production of a glycosaminoglycan derivative inhibitory to heparanase activity but without substantially anticoagulant activity. Heparanase-inhibiting glycosaminoglycan derivatives having no substantial anticoagulant activity may also be prepared from heparin, for example, by: at least partial N-desulfation and then N-acetylation; or at least partial N-, O-desulfation followed by N-resulfation.

15

FIGURE 1: Monomeric structural formulas which represent derivatives of heparin used in this study (actual structures obtained by derivatization are averages of monomeric units shown). a, Intact heparin; b, Partially N-desulfated heparin; c, completely N-desulfated heparin; d, N-acetylated N-desulfated heparin; e, N- and O-desulfated heparin; f, N-acetylated N- and O-desulfated heparin; g, N-resulfated N- and O-desulfated heparin; h, carboxy-reduced heparin.

FIGURE 2: Cellulose acetate sheet electrophoresis of chemically modified heparins. The sheet is 7.6 cm in total length, with 1.5 cm of stacking area blotted with water and 5.0 cm of separating area blotted with a 0.5M pyridine/0.5M acetic acid, pH 5.0 buffer. The electrodes are in the same buffer. Electrophoresis is performed under constant voltage (12 V/cm) at 4°C for 45 min. After electrophoresis, the sheet was stained with toluidine blue (0.1% toluidine blue in 1% acetic acid) and destained with 1% acetic acid.

FIGURE 3: HPLC of chemically modified heparin on a single 2.7 x 75 cm column of Fractogel TSK HW-55(S) (Irimura et al., 1983). Elution was accomplished with 0.2M sodium chloride at a flow rate of 0.75 ml/min. During the separation the column was kept at 55°C by a water jacket. Each sample was dissolved in water (5 ug/ml) and 25 µl was injected. Patterns a-h indicate the analytical results of derivatized heparins shown in Figure 1.

FIGURE 4: Results of HPLC assay of B16 melanoma heparanase activity in the presence of chemically modified heparin derivatives. A high-speed gel-permeation chromatography system was equipped with a 0.70 x 75 cm column of Fractogel TSK-HW55(S). Elution was performed with 0.2 M NaCl at a flow rate of 1.0 ml/min at 55°C. Each 0.5-ml fraction corresponding to 30-sec elution was collected, mixed with 3 ml of Hydrofluor and its radioactivity determined. Panel A, ¹⁴C-HS was incubated with heat-inactivated crude heparanase (10⁶B16-BL6-melanoma cell equivalents) at 37°C for 6 h, and analyzed. B, same incubation as A except that the enzyme was not heat-inactivated. C, same incubation and analysis as B, but 1 mg/ml of heparin was added to the incubation mixture. D, same incubation and analysis as B, but with 1 mg/ml of completely N-desulfated heparin added to the incubation mixture. E, same incubation and analysis as B, with 1 mg/ml N- and O-desulfated heparin added to the mixture. F, same as B, but with 1 mg/ml of N-resulfated N- and O-desulfated heparin added.

FIGURE 5: Inhibitory activities of chemically modified heparins on release of ³H-acetylated HS fragments by metastatic B16 melanoma heparanase. The measurements were performed by incubating B16-BL6 melanoma heparanase. The measurements were performed by incubating B16-BL6

-15-

melanoma cell lysates equivalent to 10^6 cells with a suspension of ^3H -HS-agarose beads prepared by partial N-desulfation and ^3H -acetylation (approximately 20% suspension), and various concentrations of heparanase inhibitors at 37°C for 24 hr with gentle mixing. Panels a-h indicate the results using the 8 different chemically modified heparins as indicated in Figures 1 and 2. The inhibitory activities are indicated by the percent release of HS fragments compared to the release without added inhibitors.

FIGURE 6: Effects of chemically modified heparins on the release of ^{35}S labeled substances from the extracellular matrix of mouse lung capillary endothelial cells in vitro. Heparin and some chemically modified heparins induced non-enzymatic release of ^{35}S labeled materials in the absence of B16 melanoma cells, while they inhibited the degradation of these macro molecules caused by B16 melanoma cells. (See: Nakajima et al., Science 220:611-613, 1983, for the methods.)

FIGURE 7: Effects of chemically modified heparins on the incorporation of ^3H -thymidine to B16-BL6 melanoma cells grown in tissue culture. (See: Irimura et al., (1981) Cancer Res. 41:3411-3418, for the methods.)

FIGURE 8: Adhesion of B16 melanoma cells to bovine aortic endothelial cell monolayers. (a) B16 melanoma cells were pretreated with modified heparins at room temperature for 2 hrs, and the adhesion assays were performed in the continuous presence of these compounds at 37°C . (b) Endothelial cells were pretreated with modified heparins at 37°C for 2 hrs, then adhesion assays were performed. (See Nicolson and Custead, (1985) Cancer Res. 45:331-336, for the methods)

FIGURE 9: Effects of modified heparins on the adhesion of B16-F10 melanoma cells to the syngenic mouse lung tissues in vitro. Primary organ cultures of minced mouse lung tissues were mixed with melanoma cell suspensions and incubated at 37°C under gentle gyration. The attached melanoma cells to the lung tissues were histologically quantitated after sectioning. (See Nicolson et al., (1985) *Invasion Metastasis*, 5:144-158, for detailed methods.)

FIGURE 10: Effects of modified heparins on the B16-F10 melanoma cell-invasion of isolated mouse lung tissues in vitro. The experimental conditions were the same as the adhesion experiments. The invaded cells were counted on 4 μ m sections under a light microscope. (See Nicolson et al., (1985) *Invasion Metastasis*, 5:144-158, for detailed methods.)

Heparanase from metastatic melanoma cells is an endo-beta-glucuronidase which is specific for HS (Irimura et al., (1983b), *Gann Monogr. Cancer Res.*, V 29, pp 35-46; Nakajima et al., (1984) *J. Biol. Chem.*, V 259, pp 2283-2290). Although heparin is structurally and biosynthetically related to HS, it is a poor substrate for heparanase, and it interferes with HS degradation (Nakajima et al., (1984)). Structural differences between heparin and HS are based primarily on the degrees of sulfation of glucosamine residues, and the relative contents of iduronic acid. The heparanase-inhibitory activity of heparin should be determined, therefore, by its sulfate as well as its carboxy groups. If one of these group were responsible for heparanase inhibition, this information should be useful in developing specific heparanase inhibitors. Furthermore, since sulfamine and O-sulfate groups of glucosamine, O-sulfate groups of iduronic acid, and carboxy groups of uronic acid are

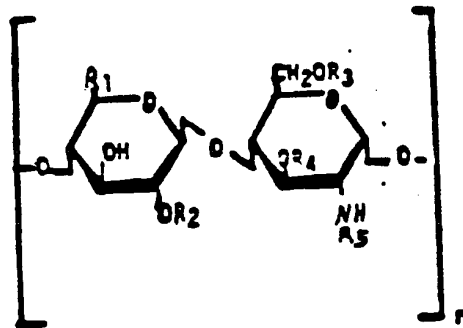
essential for heparin's anticoagulation activities, some of the chemical modifications should produce heparin derivatives that inhibit heparanase activity but are not anticoagulants.

5

The glycosaminoglycan derivatives of the present invention are derivatives of heparin or heparin analogs which are effective inhibitors of heparanase while being substantially devoid of the anticoagulant activity that is characteristic of heparin. They are useful for inhibition of tumor metastases. In particular, preferred compounds of the invention are glycosaminoglycan derivatives of the following structure:

15

20



Where

25

R_1 is $-\text{COOH}$ or $-\text{CH}_2\text{OH}$ and the

configuration of the carbon atom to which R_1 is

30

bound is D or L;

R_2 is $-\text{H}$ or $-\text{SO}_3^-$;

R_3 is $-\text{H}$ or $-\text{SO}_3^-$;

35

R_4 is $-\text{H}$ or $-\text{SO}_3^-$;

-18-

R_5 is $-\text{SO}_3^-$ or $-\text{CO}-\text{CH}_3$; and

$n = 3-30$;

5 provided that: when R_1 is $-\text{CH}_2\text{OH}$, at least 50% of R_2 , R_3 and R_5 is $-\text{SO}_3^-$;

when R_1 is $-\text{COOH}$, and R_2 , R_3 and R_4 are $-\text{H}$, R_5 is $-\text{SO}_3^-$;

10 when R_1 is $-\text{COOH}$, and at least 50% of R_2 , R_3 and R_4 is $-\text{SO}_3^-$, R_5 is $-\text{CO}-\text{CH}_3$; and

15 each of the terminal monomeric units is a repeating monomeric unit having a terminal oxygen atom bound to a blocking group.

The preferred glycosaminoglycan derivatives of the invention include those having a monomeric repeating structure as given above and may be utilized as salts. Pharmaceutically acceptable salts, for example, potassium or magnesium salts, of the glycosaminoglycan derivatives described above are also suitable for use in the method of the invention and are within the scope of the present invention.

25 The glycosaminoglycan derivatives of the invention, which are oligomeric and/or polymeric forms of the monomeric repeating structure given above, suitably have a molecular weight between about 1,000 and about 15,000. These glycosaminoglycan derivatives have a preferred molecular weight between about 10,000 and about 12,500.

35 The present invention involves a method for inhibiting the spread of cancer. This method relates to the inhibition of glycosaminoglycan-degrading enzymes

-19-

which may play significant roles in the invasive or metastatic behavior of tumors. Described herein are newly devised inhibitors for heparanase, an enzyme often found in tumors, which degrades glycosaminoglycans of the extracellular matrix or basement membrane such as heparan sulfate. Such degradation is involved with tumor penetration of and attachment to biological structures.

Heparin is a preferred precursor for the synthesis of these cancer-inhibiting substances. Other glycosaminoglycans may be used as these substances or precursors to such substances. These other glycosaminoglycans may include, for example, hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate or heparan sulfate. Functionally, these cancer-inhibiting substances have qualities of impeding the activity of glycosaminoglycan-degrading enzymes and being substantially devoid of anticoagulant activity. A generally desirable chemical property of usable cancer-inhibiting glycosaminoglycan substances is the presence of sulfated amino groups.

In a general sense the cancer-inhibiting substances of the present invention may be synthesized by, for example:

(1) First, identifying a material, usually a sulfated glycosaminoglycan, which inhibits heparanase and has anticoagulation activity. Then, chemically altering this material to substantially remove anticoagulation activity while enhancing or at least not removing heparanase-inhibitory activity. Many of the Examples appended hereto illustrate this approach; or,

(2) First, obtaining a glycosaminoglycan which substantially neither has anticoagulant activity nor

inhibits heparanase. Then, chemically altering the glycosaminoglycan, for example by sulfation of amino groups, to produce a glycosaminoglycan derivative having heparanase inhibitory activity but being substantially devoid of anticoagulant activity.

Chemically modified substances may be assayed by methods described herein to follow effects upon anticoagulant activity and heparanase-inhibitory activity. When derivatives having these properties are non-toxic at levels adequate to inhibit heparanase activity are obtained, they would be suitable for the practice of the methods of the present invention.

In chemotherapeutic usage, the cancer-inhibiting substances of the present invention will be administered parenterally at dosages between about 30 mg/day and about 250 mg/day, preferably between about 30 mg/day and about 100 mg/day. However, some variation in dosage will necessarily occur depending on the condition of the subject being treated and the potency of the particular substance. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In breast cancer, adjuvant chemotherapy, with or without hormone treatment, has been used for treatment of operable breast cancer (Fisher et al., NCI Monograph, V 1, pp 35-43, 1986). At M.D. Anderson Hospital, adjuvant chemotherapy trials have utilized, as a minimum treatment protocol, a program consisting of 5-fluorouracil, doxorubicin, and cyclophosphamid (Buzdar et al., NCI Monograph, V 1, pp 81-85, 1986). In more recent studies cycles of chemotherapy were repeated at 28 day intervals using continuous infusion pumps to administer the chemotherapeutic agents. In breast cancer, administration of

-21-

non-anticoagulation heparin derivatives may be used at two times during therapy: (1) during surgery of the primary tumor and post-operatively to inhibit the dissemination of cancer cells caused by surgical manipulation, and (2) during the continuous infusion of
5 chemotherapeutic drugs, to prevent tumor cell dissemination in the blood and capillary lodgement caused by drug damage of normal tissues, such as endothelial cells (Nicolson et al., Cancer Res., V 45, pp 331-336,
10 1985). The heparin derivatives would be administered intravenously in amounts between about 30 mg/day and about 250 mg/day, preferably between 30 mg/day and about 100 mg/day. However, some variation in dosage will necessarily occur depending upon the condition of the
15 subject being treated and the potency of the particular substance. The physician responsible for administration will determine the appropriate dose for the individual subject.

20 In malignant melanoma the following example of adjuvant therapy has been used to treat metastatic disease. Patients have been treated with an adjuvant sequence of cisplatin, vinblastine and bleomycin intravenously every three weeks for a total of three cycles
25 (Johnson et al., Cancer Treatment Rep., V 69, pp 821-824, 1985). The heparin derivatives would be administered intravenously, as described above, during administration of the chemotherapeutic agents.

30 The novel compounds of the invention may be administered alone or in combination with pharmaceutically acceptable carriers and will typically be formulated in a unit injectable dosage form (solution, suspension, emulsion), preferably in a pharmaceutically acceptable
35 carrier which is inherently non-toxic and non-antigenic. In this regard, suitable carriers for formulations in

accordance with the invention include saline, Ringer's solution, mannitol, dextrose solution, and normal serum albumin. Non-aqueous carriers such as fixed oils and ethyl oleate may also be used. The carrier may also
5 contain small amounts of additives such as substances which enhance isotonicity and chemical stability, for example, buffers and preservatives. In any case, the formulations in accordance with the invention shall contain an effective amount of glycosaminoglycan
10 derivative to impede the spread of cancer cells by metastases or invasion.

These aqueous solutions are especially suitable for parenteral administration. In this connection, the
15 sterile aqueous media employed are all readily available by standard techniques known to those skilled in the art. Treatment in accordance with the invention is performed in a manner such that a maximal inhibition of tumor spread will occur. This will involve the
20 intraperitoneal, intravenous, intraarterial or intramuscular injection of the maximum tolerated dose of glycosaminoglycan derivative which is effective in invoking the desired inhibition of cancer cell spread in the patient.

25 The following examples are presented to describe preferred embodiments and utilities of the present invention and are not meant to limit the present invention unless otherwise stated in the claims appended
30 hereto.

EXAMPLE 1

MATERIALS AND METHODS

5 Chemical Modification of Heparin. Chemical
modifications and the resultant compounds used in this
study are schematically shown in Figure 1. Heparin from
porcine intestinal mucosa (sodium salt) was purchased
from Sigma Chemical Co. (St. Louis, MO.). One gram of
10 heparin was dissolved in 30 ml of water and applied to a
1.5 x 8 cm column of Dowex 50W X 8, 50-100 mesh (H⁺ form)
(BioRad, Richmond, CA) at 4°C. The pass-through fraction
eluted with water was neutralized immediately with
pyridine and the pH adjusted to between 6 and 7
15 (Nagasawa, et al., (1980a) Meth. Carbohyd. Chem., V 8, pp
287-289). After dialysis against water, the heparin
pyridinium salt was collected by lyophilization. Partial
N-desulfation, complete N-desulfation, and complete N-
and O-desulfation, starting with 100 mg each of heparin
20 pyridinium salts, were achieved by solvolysis in 10 ml of
dimethylsulfoxide (ACS grade, Fisher Scientific, Fair
Lawn, NJ.) containing water or methanol as described by
Nagasawa, et al. *ibid* and Nagasawa, et al. (1980b) Meth.
Carbohyd. Chem., V 8, pp 291-294. Reaction conditions
25 were 10% water in dimethyl sulfoxide at 20°C for 1 h for
partial N-desulfation, 10% water in dimethyl sulfoxide at
80°C for 5 hr for complete N-desulfation, and 10%
anhydrous methanol in dimethylsulfoxide at 80°C for 18 h
for complete N- and O-desulfation. After these
30 reactions, the mixtures were cooled and 1 M sodium
hydroxide was added to adjust the pH to between 8.5 and
9.5; the mixtures were dialyzed against running tap water
and then against distilled water.

35 N-acetylation of N-desulfated and N- and O-
desulfated heparin was performed with acetic anhydride

-24-

under alkaline conditions as follows: fifty milligrams of modified heparin were dissolved in 5 ml of 4.5 M sodium acetate plus 1.0 ml of methanol and added to five portions of 1.0 ml of acetic anhydride at 10 min intervals. After 1 hr incubation with occasional mixing, the reaction mixture was dialyzed against running tap water, then distilled water, and was finally lyophilized.

The N-resulfation reaction was performed by sulfation with a triethylamine sulfur trioxide complex prepared according to Cherniak and Davidson's (1964) method (J. Biol. Chem., V 239, pp 2986-2990). Complete N- and O-desulfated heparin (50 mg) was dissolved in 2 ml of 1.0 M sodium carbonate and added to 50 mg of triethylamine sulfur trioxide. The atmosphere was replaced with nitrogen, and the mixture was heated at 50°C for 24 h with occasional agitation. The resulfated heparin was dialyzed against running tap water, then distilled water, and finally lyophilized. Carboxyl-reduced heparin was prepared from sodium salt of porcine intestinal mucosa heparin as described by Taylor et al. (1976) (Meth. Carbohyd. Chem., V 7, pp 149-171) by use of sodium borohydride (Aldrich Chemical Co., Milwaukee, WI) and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide-hydrochloride (Fluka Chemical Co., Hauppauge, N.Y.).

Analytical Procedures. The homogeneity of the chemically modified heparins was assessed by cellulose acetate electrophoresis and high-speed gel-permeation chromatography. Electrophoresis was performed on a 7.6 x 7.6 cm Titan III Zip Zone cellulose acetate sheet (Helena Laboratories, Beaumont, TX) in 0.5 M/0.5 M pyridine-acetate buffer, pH 5.0 (Hata et al., (1979), Anal. Biochem., V 45, pp 412-469). Each 1 µl sample was applied to 2.0 cm wide stacking zone which was blotted with water before the sample application (Cappeletti et

-25-

al., (1979) Anal. Biochem., V 99, pp 311-320).

Electrophoresis was carried out under constant voltage (75V) for 45 min. During the run the sheet was immersed in petroleum ether cooled under ice. The cellulose acetate sheet was then stained with 0.1% toluidine blue O in 1% acetic acid and destained with 1% acetic acid.

High-speed gel permeation chromatography was performed as described previously (Irimura et al., (1983a) Anal. Biochem., V 130, pp 461-468) using a Constametric III (LDC-Milton Roy, Riviera Beach, FL) with a single 0.7 x 75 cm stainless steel column packed with Fractogel TSK HW-55(S). Elution was accomplished with 0.2 M sodium chloride at flow rates of 1.0 ml/min or 0.75 ml/min. Absorption at 210 nm was monitored for the analogs of chemically modified heparin. For the qualitative examination of radiolabeled HS-degradation products, each fraction corresponding to 30-sec elution was collected into plastic scintillation vials, and radioactivity in each vial was determined after the fraction was mixed with 3.0 ml of Hydrofluor (National Diagnostics, Somerville, NJ).

Source of Heparanase. Highly invasive mouse B16 melanoma (B16-BL6) cells were provided by Dr. I. J. Fidler, (M.D. Anderson Hospital, Houston, TX) and were cultured as previously described (Irimura et al., (1983a) and Irimura et al. (1984)). Cell extracts were prepared in 5 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 50 μ M calcium chloride, 10 μ M phenylmethylsulfonylfluoride, and 0.2% Nonidet P-40 (Irimura et al., (1983). The melanoma extracts were stored frozen at -80°C and used as crude heparanase.

Radio-Labeling of HS. 14 C- or 3 H-heparan sulfate was prepared by chemical deacetylation and radioactive

reacetylation. Nine milligrams of bovine lung HS were dried with 0.1 mg of hydrazine sulfate over phosphorous pentoxide under vacuum at 50°C for 48 h. Anhydrous hydrazine (0.5 mg, Pierce Chemical, Rockford, IL) was added to the dried HS and the mixture heated in a tightly screwed tube under nitrogen atmosphere at 100°C for 1 h. After the reaction, the hydrazine was removed by repeated evaporation with toluene over sulfuric acid desiccant under vacuum conditions. To separate deacetylated HS from residual reagents and partial degradation products, the completely dried residue was dissolved in 0.5 ml of water and subjected to gel filtration on a 0.8 x 30 cm column of BioGel P-10 and elution with distilled water. The void volume fraction was collected and lyophilized, and the yield by weight was about 60%. N-deacetylated HS was then N-acetylated with 50 uCi of ^{14}C -acetic anhydride (10 mCi/mmol: New England Nuclear, Boston, MA.) or 5 mCi ^3H -acetic anhydride (400 mCi/mmol: NEN) in 0.5 ml of 4 M sodium acetate for 18 hrs. N-acetylation was completed by addition of 0.1 ml of unlabeled acetic anhydride to the reaction mixture and incubation for 1 h. ^{14}C - or ^3H -HS was purified on a BioGel P-10 column as described above.

High-Performance Liquid Chromatography [HPLC] Assay for Heparanase. Fifty microliters of melanoma extract (equivalent to 10^6 cells) were mixed with chemically modified heparin (5 mg/ml in water), 50 μl of 4 X heparanase assay buffer (0.4 sodium phosphate buffer, pH 5.8, containing 0.4% Triton X-100, 0.6 M sodium chloride, and 0.4 % sodium azide) and about 3000 cpm of ^{14}C -HS. Incubation was performed at 37°C with continuous gentle mixing for 6 h. The reaction mixture was placed on ice, 20 μl of 50% trichloroacetic acid was added, and incubation continued on ice for 10 min. After centrifugation at 9000 x G for 5 min in a Microfuge B

(Beckman Instruments, Irvine, CA), the supernatant was injected into the gel-permeation chromatography system and analyzed as described above (Irimura et al., (1983a)).

5

Preparation of Solid-phase Substrates for Heparanase and Inhibitor Assays. For the solid-phase heparanase assay, ^3H -HS was aminated at the reducing terminal with 2 M ammonium acetate in the presence of 0.4 M sodium cyanoborohydride in 50% methanol, at 50°C for 6 days. Aminated ^3H -HS was purified by gel filtration as described above, and the resulting solution was diluted to 0.1 M in sodium carbonate. To 10^6 cpm of aminated ^3H -HS, 1.0 ml of Affi-Gel 15 (Bio Rad) gel beads was added after they were washed with isopropanol and chilled water. The coupling reaction was continued at 4°C for 48 h with continuous agitation. The gel beads were then reacted with 0.1 M glycine monomethyl ester dissolved in 0.1 M sodium carbonate for 1 h at room temperature and then washed with 4 M sodium chloride repeatedly to remove noncovalently attached ^3H -HS from the beads.

Solid-phase Assays for Heparanase and Heparanase Inhibitors. ^3H -HS-agarose was suspended in Dulbecco's phosphate buffered saline (DPBS) at about 20% (v/v). The incubation conditions for the solid-phase assay were identical to those of the HPLC assay, except that 75 μl of the ^3H -HS-agarose suspension was used instead of the HS so that the incubation mixture consisted of B16 melanoma extract, chemically modified heparins, 4X heparanase assay buffer, and ^3H -HS-agarose suspension. After incubation, the reaction mixture was placed on ice, chilled 5% trichloroacetic acid (50 μl) added, the mixture incubated for 10 min, and centrifuged. Radioactivity in the supernatant and the pellet was determined separately after mixing with Hydrofluor.

Experimental Metastatic Lung Colonization of Melanoma Cells. Male C57BL/6 mice 4 to 6 weeks old were obtained from Charles River, Inc. (Kingston, MD) and quarantined for 2 weeks. Animals were fed normal rodent chow and unchlorinated spring water. B16-BL6 cells were grown to subconfluence, detached from plastic dishes by incubating in 2 mM EDTA, 0.14 M NaCl and 10 mM sodium phosphate-buffer, pH 7.4, for 5-10 min, and suspended in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium. The cells were incubated with heparin, N-acetylated N-desulfated heparin, N-resulfated N and O-desulfated heparin or carboxy-reduced heparin (each 0.5 mg/ml in the media described above) at 4°C for 2 hrs. Treated or untreated cells ($5 \times 10^4/0.1$ ml) were injected intravenously to each mouse. Mice were killed 20 days later and autopsied. The numbers of pulmonary tumor nodules were counted after the lung was perfused via the trachea with 4% formalin in Dulbecco's phosphate buffered saline. Extrapulmonary tumor formation was assessed in each animal and recorded.

EXAMPLE 2

CHEMICALLY MODIFIED HEPARINS

Chemical Modifications. Porcine intestinal mucosal heparin was chemically modified as schematically shown by the representative structures in Figure 1. When the electrophoretic mobilities of these substances in pyridine/acetate buffer on cellulose acetate sheets were compared (Figure 2), all of the chemical modified heparins migrated more slowly than intact heparin under the electrophoretic condition used. N- and O-desulfated heparins remained at the top of stacking gel and did not stain intensely with Toluidine blue O. The migration

distances of the modified heparins were, in order from shortest to longest: N- and O-desulfated heparin, N-acetylated N- and O-desulfated heparin, N-desulfated heparin, N-resulfated N- and O-desulfated heparin, N-acetylated N-desulfated heparin, partially N-desulfated heparin, and carboxy-reduced heparin. High-speed gel-permeation chromatography of these substances was performed as described in Example 1 (Figure 3). A slight change in the apparent molecular size was observed, probably as a result of detachment and reattachment of the relatively bulky side groups to the heparin chain. Degradation products were not observed in any of the modified heparin preparations as determined by high speed gel permeation chromatography

15

EXAMPLE 3

HPLC ASSAY OF HEPARANASE INHIBITORY
ACTIVITIES OF CHEMICALLY MODIFIED HEPARINS

Intact heparin, N-desulfated heparin, N- and O-desulfated heparin, and N-sulfated N- and O-desulfated heparin (4 mg/ml dissolved in water) were mixed with 50 μ l of crude heparanase and 14 C-HS as described above. The elution profiles of the radioactivity on high-speed gel-permeation chromatography are shown in Figure 4. The elution profile of 14 C-HS incubated with heat-inactivated (100°C for 5 min) heparanase was identical to that of untreated 14 C-HS, which eluted at the position corresponding to an approximate M_r of 34,000. After incubation with heparanase, the average apparent M_r decreased to 6,000. In the presence of heparin, no degradation was observed. N-desulfated heparin or N- and O-desulfated heparin failed to inhibit degradation of HS by heparanase. After N-resulfation of N- and O-desulfated

35

heparin, the heparanase inhibitory activity was partially restored. O-sulfate groups on the 3 or 6 position of glucosamine, as well as on the 2 position of iduronic acid, seem less essential to heparanase inhibition, provided that the amino groups are completely resulfated.

EXAMPLE 4

SOLID-PHASE ASSAY FOR HEPARANASE

Since heparanase is an endoglycosidase that produces relatively large fragments of HS, rapid isolation of the fragmented HS from intact HS is necessary for the quantitative assay. Therefore, HS chains were immobilized at their ends to a solid-phase support, such as small beads. First, ^{35}S -HS (purified from the subendothelial matrix in cell culture (Wang et al., (1985) Eur. J. Biochem., V 153, pp 125-130) were immobilized onto Affi-Gel 15, since this HS preparation contained amino acid residues at its reducing terminal end. With use of this substrate, however, the proportion of ^{35}S -labeled materials released from the beads by crude heparanase was negligible, and this method was not found likely to be useful. Next, chemically labeled and modified HS was immobilized on beads. Deacetylation of HS was achieved by hydrazinolysis. Lower temperatures and shorter reaction times than the usual hydrazinolysis reactions of glycoproteins (Fukuda et al., (1976) J. Biochem., V 80, pp 1223-1232); Irimura et al., (1981) Biochem, V 20, pp 560-566) were chosen because of possible cleavage of uronosyl-glucosaminide through these amino groups. Resultant N-deacetylated heparin was labeled by N- ^{14}C - or ^3H -acetylation. It appeared to be important to subsequently block all free amino groups by acetylation with non-radioactive acetic anhydride, which was done. Radio-labeled HS was aminated exclusively at its reducing

-31-

terminal by reductive amination, and coupled to Affi-Gel 15 linkage under alkaline conditions. The proportion of labeled HS coupled to agarose beads fluctuated between 50 and 80% calculated from the amount of material used for the amination reaction. As a heparanase substrate, partially N-desulfated and N- ^3H - or ^{14}C -acetylated HS are also as useful as deacetylated and reacetylated HS. Partial N-desulfation of HS was achieved by the same reaction conditions used for partial N-desulfation of heparin described under Materials and Methods.

EXAMPLE 5

HEPARANASE INHIBITION BY CHEMICALLY MODIFIED
HEPARINS AS MEASURED BY A SOLID-PHASE ASSAY

The dose response curves of the inhibitory effects of chemically modified heparins are shown in Figure 5. The use of i) intact, ii) N-desulfated, iii) N- and O-desulfated and iv) N-resulfated, N- and O-desulfated heparin produced results consistent with those of the HPLC assay. N-desulfated and N- and O-desulfated heparin failed to show any inhibitory activity, whereas partial restoration of activity was obtained by the addition of sulfamino groups (Figure 5g). Interestingly, when the exposed amino groups formed by N-desulfation were acetylated, heparanase inhibitory activity was partially restored (Figure 5d). N-Acetylation of N- and O-desulfated heparin did not, however, restore the inhibitory activity (Figure 5f). These results indicated that sulfamino groups, although they affected heparanase inhibition, were not essential for it. Removal of sulfamino groups, but with intact O-sulfate groups of heparin resulted in inhibitory activity, provided that the exposed amino groups are blocked by acetylation.

-32-

Carboxy-reduced heparin was shown to possess weaker inhibitory activity than heparin, which indicated that heparin carboxy groups were necessary but not essential, for the full inhibitory activity. Similar observations have been described concerning heparin's stimulatory activity on the growth of smooth muscle cells (Castellot et al., (1984) J. Cell. Physiol., V 120, pp 315-320).

EXAMPLE 6

MODIFIED HEPARINS AS ANTICOAGULANTS

Anticoagulation activities of N-acetylated N-desulfated heparin, N-resulfated N- and O-desulfated heparin, and carboxyl-reduced heparin were measured by the USP standard assay. The anticoagulation activities of these three compounds were each less than 1% of the anticoagulation activity of unmodified heparin.

EXAMPLE 7

EFFECTS OF MODIFIED HEPARINS ON
EXPERIMENTAL BLOOD-BORNE LUNG
COLONIZATION OF B16 MELANOMA IN MICE

As shown in Table 1, the number of visible melanoma colonies in lung 20 days after the injection of the tumor cells was significantly reduced by preincubation of the cells with intact or chemically modified heparin. The effect of intact heparin was greater, probably because additional factors such as inhibition of melanoma-induced platelet aggregation were involved. Since these three chemically modified heparins do not possess anticoagulation activity, the inhibition of melanoma lung colonization

tion appeared to be due to inhibition of melanoma
heparanase.

TABLE 1
THE EFFECTS OF CHEMICALLY-MODIFIED HEPARINS ON BLOOD-BORNE LUNG
COLONIZATION OF B16-BL6 MELANOMA CELLS IN MICE

Treatment ^a	Exp 1 (9 mice/group) Number of colonies (median)	Exp 2 (9 mice/group) Number of colonies (median)
None	8, 20, 28, 33, 75, 85, 106, 116, 193, (75)	0, 1, 26, 48, 75, 163, 193, 200+, 200+, (75)
Heparin	0, 1, 8, 9, 16, 18, 21, 82, 174, (16)	0, 0, 0, 0, 0, 1, 1, 2, 12, (0)
N-acetylated-N-de- sulfated heparin	11, 19, 22, 23, 43, 89, 109, 199, (43)	0, 1, 2, 3, 5, 5, 25, 37, 200+, (5)
N-resulfated-N-, O- desulfated heparin		0, 0, 2, 5, 8, 13, 20, 90, 200+, (8)
Carboxy-reduced heparin	0, 15, 25, 25, 29, 36, 45, 46, 53, (29)	7, 13, 42, 49, 51, 55, 58, 89, 120, (51)

^aThe cells were incubated with chemically modified heparins (500 ug/ml) at 4°C for 2 hr before injection.

-35-

EXAMPLE 8

EFFECTS OF MODIFIED HEPARINS ON THE DEGRADATION OF LUNG
CAPILLARY ENDOTHELIAL MATRIX BY MELANOMA CELLS IN VITRO

5

Effects of chemically modified heparins on the release of ^{35}S -labeled polysaccharides from the extracellular matrix of mouse lung endothelial cells were studied in vitro. The endothelial cells isolated from mouse lung capillary were grown in the presence of ^{35}S -sulfate, and the extracellular matrix-like material was isolated on multiwell plastic tissue culture plates by hypotonic lysis of the cells. The matrices (1 cm diameter) were incubated with mouse B16-BL6 melanoma at 37°C for 18 hours. The released radioactivity was counted.

10

15

As shown in the Figure 6, N-resulfated- N-, O-desulfated heparin and carboxyl-reduced heparin effectively blocked the degradation of sulfated molecules, most likely heparan sulfate proteoglycans, by melanoma cells. Heparin apparently induced spontaneous release of ^{35}S labeled macromolecules, while such effects were minimal with modified heparins.

20
25

EXAMPLE 9

EFFECTS OF MODIFIED HEPARINS ON THE GROWTH
OF B16 MELANOMA CELLS IN TISSUE CULTURE

5 In order to assess a possibility that the effects of
modified heparins on the blood-borne lung colonization of
B16 melanoma cells were due to their direct toxic effects
on the cells, the growth of B16 melanoma in the presence
10 or absence of modified heparins were examined in vitro.
B16-BL6 melanoma cells were seeded in multiwell tissue
culture plates at a density 10,000 per each 1 cm well in
the presence or absence of 5% fetal bovine serum. Six
hours later, modified heparins were added, then 24 hours.
15 later, ^3H thymidine was added to a final concentration 5
uCi/ml. After further 24 hour incubation,
trichloroacetic acid-insoluble materials were collected,
dissolved in 1 M sodium hydroxide solution, neutralized
and counted on a liquid scintillation counter.

20 Figure 7 shows typical data demonstrating that there
are no significant effects on the incorporation of ^3H -
thymidine to B16 cells shown by unmodified heparin N-
acetylated-N-desulfated heparin, N-resulfated- N-, O-
25 desulfated heparin or carboxyl-reduced heparin. These
data indicated that the in vivo effects of these
compounds were due to the influence on the tumor cell-
host interactions.

EXAMPLE 10

5 EFFECTS OF MODIFIED HEPARINS ON THE ADHESION OF
 B16 MELANOMA CELLS TO THE MONOLAYER AND TO THE
EXTRACELLULAR MATRIX OF LUNG CAPILLARY ENDOTHELIAL CELLS

One of the most critical steps in the tumor cell-
microvascular endothelium interaction during metastatic
lung colonization of melanoma cells is the adhesive
10 interaction of melanoma cells with endothelial cells.
The effects of modified heparins on the adhesion of B16
melanoma to the cell monolayer and to the extracellular
matrix of lung capillary endothelial cells were studied
in vitro. The endothelial cells were grown in tissue
15 culture and the extracellular matrices were isolated as
described in Example 8. Figure 8(a) shows the time
course of the adhesion of B16-BL6 cells previously
treated with modified heparins to the monolayer of
endothelial cells, in the continuous presence of modified
20 heparins. None of these compounds, including intact
heparin, significantly altered the time course of the
adhesion of B16-BL6 cells to the endothelial cell
monolayer. Prior treatment of endothelial cells for 2
hrs with these compounds also resulted in no significant
25 alteration in the adhesion (Figure 8(b)).

EXAMPLE 11

30 EFFECTS OF MODIFIED HEPARINS ON ADHESION
 TO AND INVASION OF ORGAN-CULTURED
LUNG TISSUES BY MELANOMA CELLS IN VITRO

The effects of these glycosaminoglycan derivatives on
35 organ-specific adhesion and invasion of B16 melanoma
cells were studied by using primary organ culture
techniques in vitro. Minced lung tissues (0.5-1.0 cubic
mm) from C57BL/6 mice were placed in glass vials and

incubated in HEPES-buffered Dulbecco's modified minimum essential media containing 10% fetal bovine serum and 50 ug/ml gentamycin under continuous gyration at 66 rpm. After the medium was changed to a fresh medium without gentamycin, B16-F10 melanoma cells were added at a final concentration of 100,000/ml with or without various modified heparins. After various incubation times, the tissues were fixed in buffered formalin and processed for light microscopy. The adhesion and invasion of the tumor cells were examined on 4 mm thick sections stained with hematoxylin and eosin.

As shown in Figure 9, the number of melanoma cells attaching to the surface of minced lung tissues was significantly affected by the compounds at the early stages of incubation (6 hrs), but little effect was present after 18 hrs. Heparin and modified heparins inhibited invasion of B16 melanoma cells to the lung tissues, as shown in Figure 10. These data clearly indicated that these compounds possess capacity to block lung invasion of B16 melanoma cells.

EXAMPLE 12

25

IN VIVO INHIBITION BY HEPARIN DERIVATIVES OF METASTATIC COLONIZATION BY MELANOMA CELLS

Chemically modified heparins were tested in mice as inhibitors of blood-borne lung colonization by melanoma cells. Male C57BL/6 mice 4 to 6 weeks old were obtained from Charles River, Inc. and quarantined for 2 weeks. The mice were intravenously inoculated with 5×10^4 B16-BL melanoma cells. Treated mice were intravenously injected with 0.1 ml each of heparin or chemically modified heparin (5.0 mg/ml) 4 hr before and 20 hr after inoculation with the melanoma cells. The chemically

modified heparins used were: N-acetylated N-desulfated heparin; carboxyl-reduced heparin; and N-resulfated N, O-desulfated heparin.

- 5 Mice were killed 20 days after tumor inoculation and autopsied. The numbers of pulmonary tumor colonies were counted after the lungs were perfused via the trachea with 4% formalin in Dulbecco's phosphate-buffered saline. The results of these manipulations are shown in Table 2.
- 10 As may be seen in Table 2, heparin as well as the glycosaminoglycan derivatives substantially devoid of anticoagulation activity all impeded the establishment of metastatic tumor colonies in the lungs. Although heparin itself inhibited formation of lung metastases, the
- 15 carboxyl-reduced heparin was most effective in this particular manner.

TABLE 2
EFFECTS OF CHEMICALLY MODIFIED HEPARINES
ON BLOOD-BORNE LUNG COLONIZATION OF B16
MELANOMA CELLS IN SYNGENEIC MICE

Treatment	Number of lung colonies	Median
None	3, >150, >150, >150, >150	>150
Heparin	19, 20, 29, 30, 40, 99, 100	30
N-acetylated N-desulfated heparin	4, 26, 28, 50, 60, >150, >150, >150	60
Carboxyl-reduced heparin	0, 0, 1, 3, 6, 6, 60, 61	6
N-resulfated, N, O-desulfated heparin	0, 1, 28, 28, 30, 36, 38, >150, >150	36

-41-

EXAMPLE 13

IN VIVO INHIBITION BY HEPARIN
DERIVATIVES OF METASTATIC COLONIZATION
BY MAMMARY CARCINOMA CELLS

5

Inbred 8-week-old female Fischer (F344/CDL) rats were supplied by the Charles River Breeding Laboratories (Kingston, NY). Animals were quarantined for 7 days before use and feed standard rodent chow and unchlorinated spring water *ad libitum*. They were maintained under guidelines set forth by the University of Texas M. D. Anderson Cancer Center and the Institute of Laboratory Animal Resources, National Research Council. As a source of breast carcinoma cells, a cloned line of the 13762NF rat mammary adenocarcinoma MTF7 (Neri, A., Welch, D.R., Kawaguchi, T., Nicolson, G. L. J. *Natl. Cancer Inst.*, 68:507-517, 1982) was used. The cells grown at 37°C in an atmosphere of 5% CO₂ in humidified air in an 1:1 mixture of Dulbecco's modified minimum essential media and Ham's F12 media containing 10% fetal bovine serum and no antibiotics in 100 mm-diam. tissue culture plates.

The cells used in this study were in exponential growth phase and were from passages 14 to 20 *in vitro* and were free of mycoplasma and viral contamination. Cells were detached from tissue culture plates by brief treatment with 2 mM EDTA and 0.25% trypsin, rinsed with media (1:1 mixture of Dulbecco's modified minimum essential media and Ham's F12 media) containing 10% fetal bovine serum and with serum-free media, and then suspended in serum-free media.

Unmodified and chemically modified heparins (Irimura, T., Nakajima, M., Nicolson, G. L. *Biochemistry*, 25:5322-528, 1986) were dissolved in Dulbecco's phosphate

buffered saline at a concentration 10 mg/ml. Rats (9 per group) were injected i.v. (lateral tail vein) with 0.1 ml of 10 mg/ml solution of heparin, N-acetylated-N-desulfated heparin, or carboxyl-reduced heparin. Eight hours later, the same rats were injected i.v. with 5×10^4 MTF7 mammary carcinoma cells suspended in 0.2 ml serum-free media. Sixteen hours after injections of carcinoma cells, rats were treated with unmodified or chemically modified heparin at the same dose as the previous treatment 8 hours prior to the injection of carcinoma cells.

Rats were killed 30 days after the initial injection of carcinoma cells and examined for the number of lung tumor colonies, that should represent the degree of metastatic spread of mammary carcinoma cells. The results are shown in Table 3. Although heparin itself was most effective in this experiment, the N-acetylated, N-desulfated and carboxyl-reduced heparins were also effective. The latter two derivatives are of course depleted in anticoagulation activity.

TABLE 3
EFFECT OF MODIFIED HEPARINS ON EXPERIMENTAL METASTASIS OF
RAT MAMMARY ADENOCARCINOMA MTF7

Heparin Derivative	Lung Metastases per rat	
	Number	Median No.
None	45, 47, 56, 67, 68, 71, 73, 73, 79	68
Heparin	0, 0, 0, 5, 9, 10, 27, 33, 35	9
N-Acetylated- N-desulfated heparin	8, 18, 20, 23, 29, 32, 44, 56, 62	29
Carboxyl-reduced heparin	10, 11, 14, 19, 25, 26, 39, 45, 52	25

* * * * *

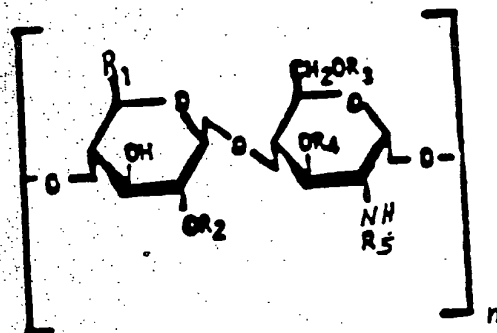
5 Changes may be made in the operation and arrangement
of the various elements, substances and procedures
described herein without departing from the concept and
scope of the invention as defined in the following
claims.

10

CLAIMS:

1. A method comprising:
 - 5 a heparin derivative which is substantially devoid of anticoagulation activity and is an effective inhibitor of heparanase activity, for use in impeding the formation of tumor metastases in a host.
- 10 2. The heparin derivatives of claim 1 defined as administered to the host in a therapeutic amount of between 30 mg/day and 250 mg/day.
- 15 3. The heparin derivative of claim 1 defined as having sulfamino or O-sulfate groups.
- 20 4. The heparin derivative of claim 1 defined as having a molecular weight between 1,000 and about 15,000.
- 25 5. The heparin derivative of claim 1 defined as having a molecular weight between 10,000 and 12,500.
- 30 6. The heparin derivative of claim 1 wherein the tumor is a melanoma or a mammary carcinoma.
7. The heparin derivative of claim 1 defined as having the formula:
- 35

-46-



where

R_1 is $-\text{COOH}$ or $-\text{CH}_2\text{OH}$ and the configuration of the carbon atom to which R_1 is bound is D or L;

R_2 is $-\text{H}$ or $-\text{SO}_3^-$

R_3 is $-\text{H}$ or $-\text{SO}_3^-$;

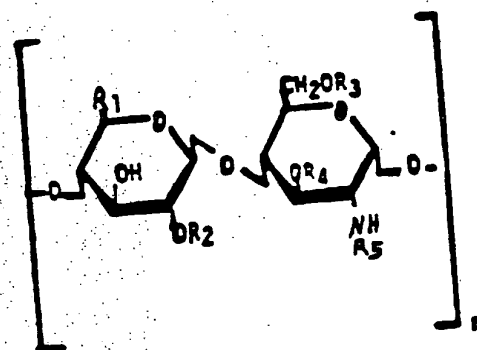
R_4 is $-\text{H}$ or $-\text{SO}_3^-$;

R_5 is $-\text{H}$, $-\text{SO}_3^-$ or $-\text{CO}-\text{CH}_3$;

n is 3 to 30; and

each terminal monomeric unit is a monomeric repeating unit with a terminal oxygen atom being bound to a blocking group.

8. The heparin derivative of claim 1 defined as having the formula:



-47-

where

R_1 is $-\text{COOH}$ or $-\text{CH}_2\text{OH}$ and the configuration of the carbon atom to which R_1 is bound is D or L;

5

R_2 is $-\text{H}$ or $-\text{SO}_3^-$;

R_3 is $-\text{H}$ or $-\text{SO}_3^-$;

R_4 is $-\text{H}$ or $-\text{SO}_3^-$;

R_5 is $-\text{SO}_3^-$ or $-\text{CO}-\text{CH}_3$; and

10

$n = 3-30$;

provided that: when R_1 is $-\text{CH}_2\text{OH}$, at least 50% of R_2 , R_3 and R_5 is $-\text{SO}_3^-$;

15

when R_1 is $-\text{COOH}$ and R_2 , R_3 and R_4 are $-\text{H}$, R_5 is $-\text{SO}_3^-$;

when R_1 is $-\text{COOH}$ and at least 50% of R_2 , R_3 and R_4 is $-\text{SO}_3^-$, R_5 is $-\text{CO}-\text{CH}_3$; and

20

each terminal monomeric unit is a monomeric repeating unit having a terminal oxygen atom bound to a blocking group.

25

9. A method for producing a metastasis-inhibiting substance comprising:

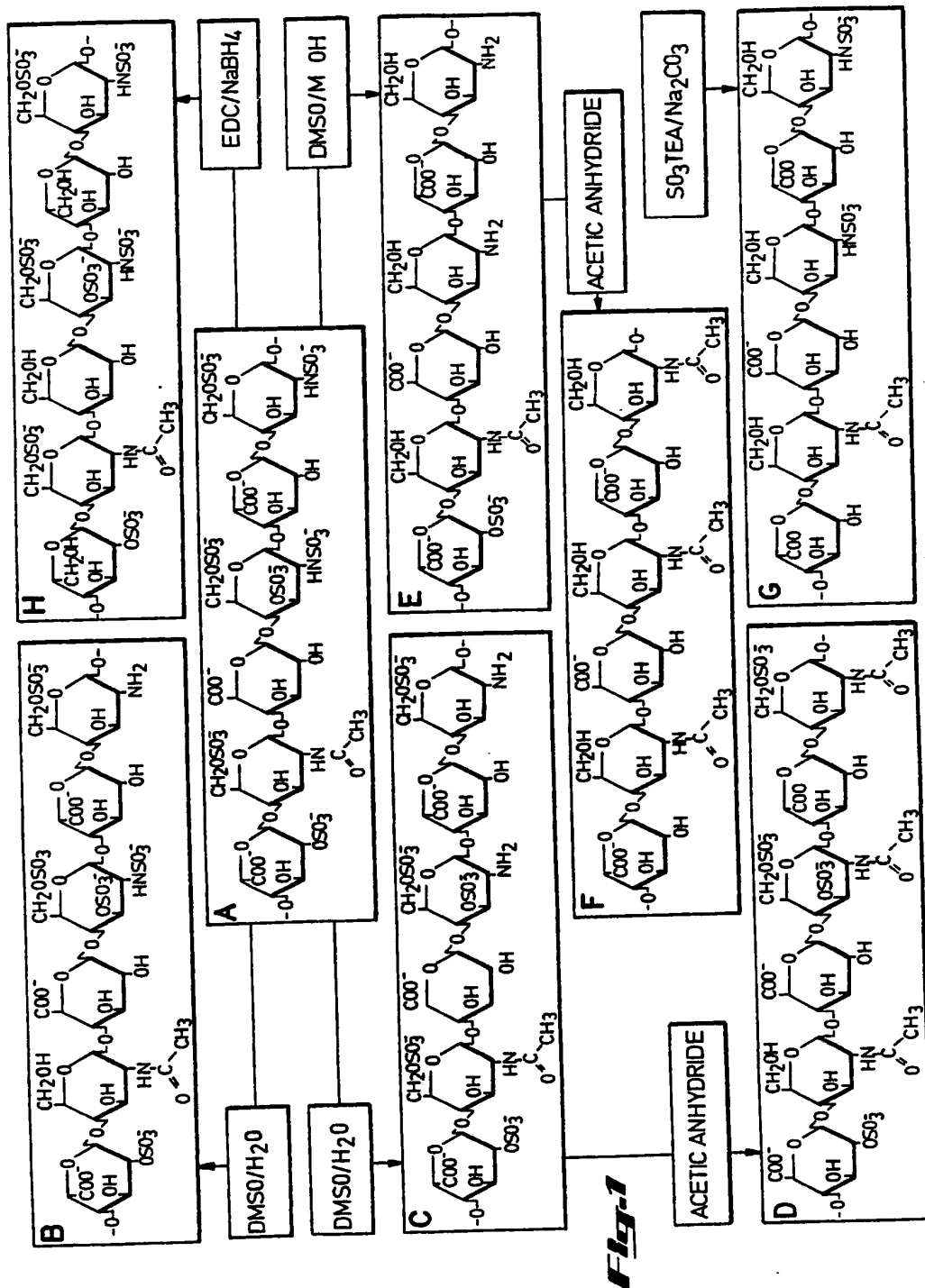
30

purifying a naturally occurring material which inhibits heparanase activity and has anticoagulation activity; and

35

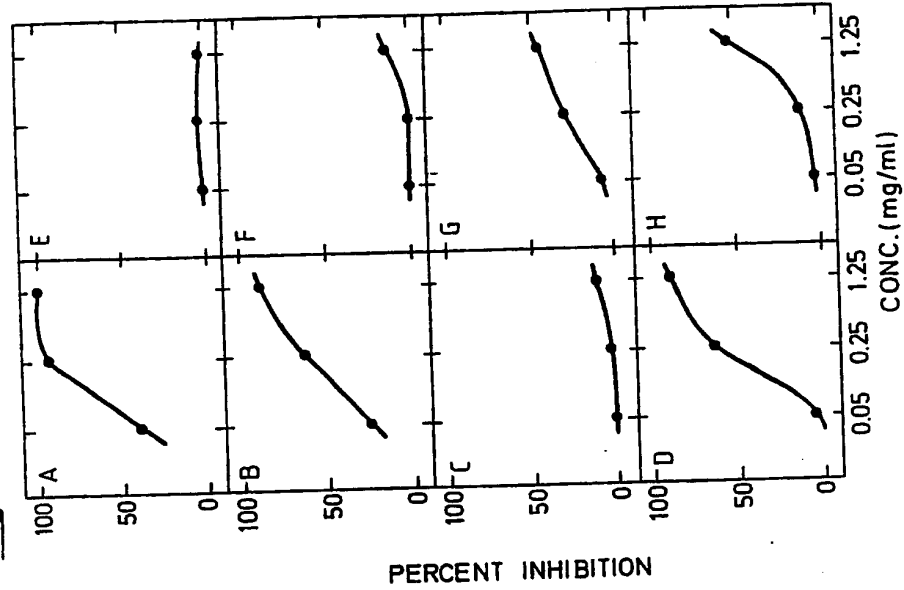
synthesizing derivatives of this material to substantially remove anticoagulation activity while enhancing or at least not removing heparanase-inhibitory activity.

10. The method of claim 9 wherein the material is a sulfated glycosaminoglycan.
- 5 11. The method of claim 9 wherein the material is heparin.
- 10 12. A method for producing a metastasis-inhibiting substance comprising:
- 15 obtaining a glycosaminoglycan which substantially has neither anticoagulant activity nor inhibits heparanase; and
- 20 synthesizing derivatives of the glycosaminoglycan by a process comprising sulfation of amino groups, to produce a glycosaminoglycan derivative having heparanase-inhibiting activity but being substantially devoid of anticoagulant activity.



2/6

Fig. 5



+

Fig. 2

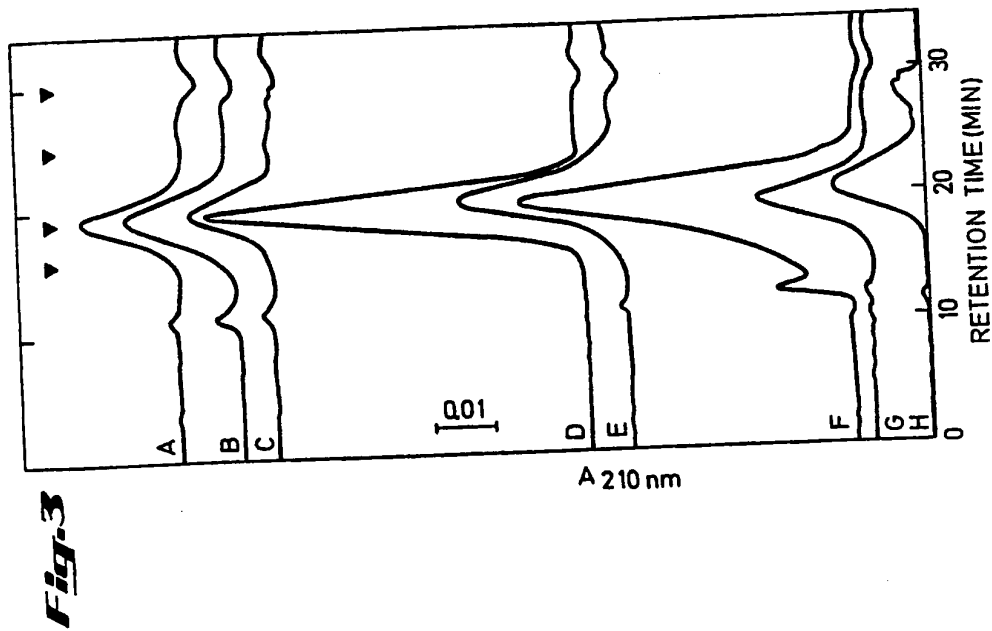
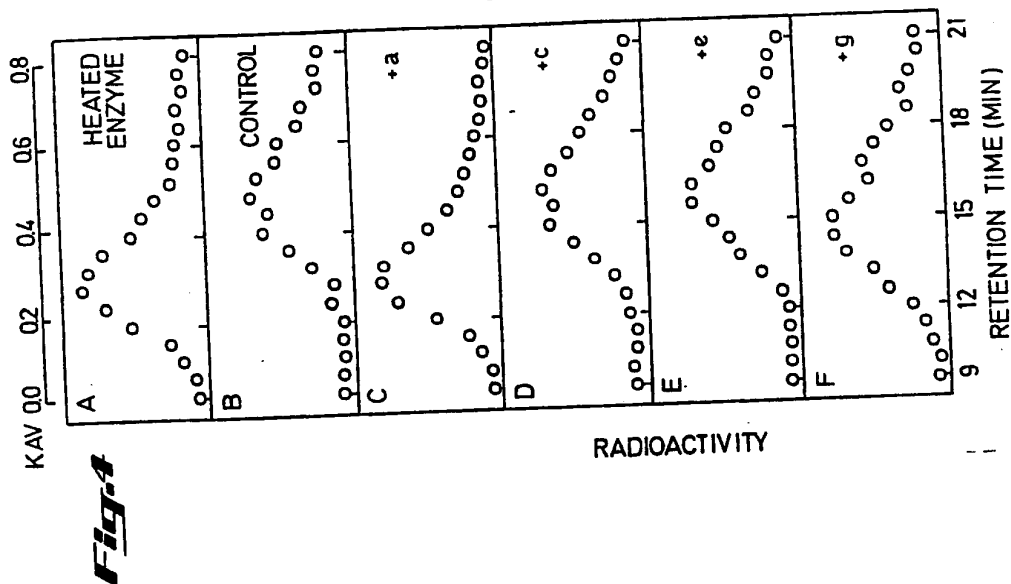


ORIGIN

- H. CARBOXYL REDUCED
- G. N-RESULFATED NO-DESULFATED
- F. N-ACETYLATED NO-DESULFATED
- E. NO-DESULFATED
- D. N-ACETYLATED N-DESULFATED
- C. COMPLETELY N-DESULFATED
- B. PARTIALLY N-DESULFATED
- A. INTACT

SUBSTITUTE SHEET

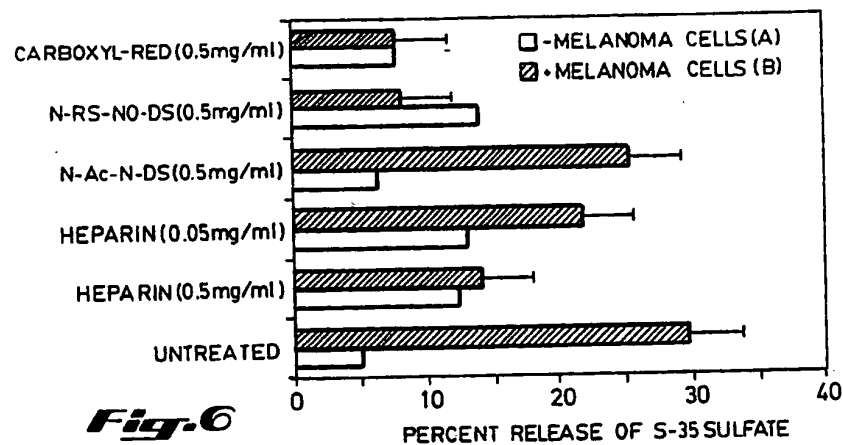
3/6



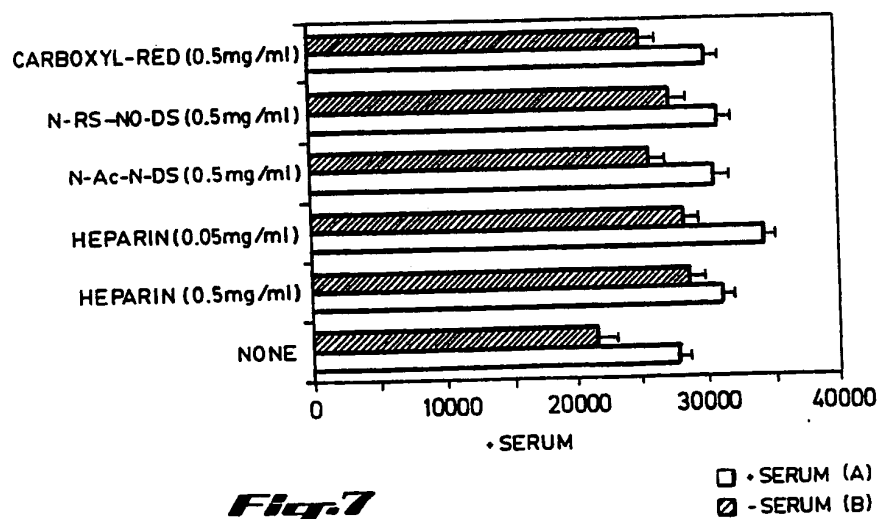
SUBSTITUTE SHEET

4/6

EFFECTS OF MODIFIED HEPARINS ON ENDOTHELIAL MATRIX DEGRADATION

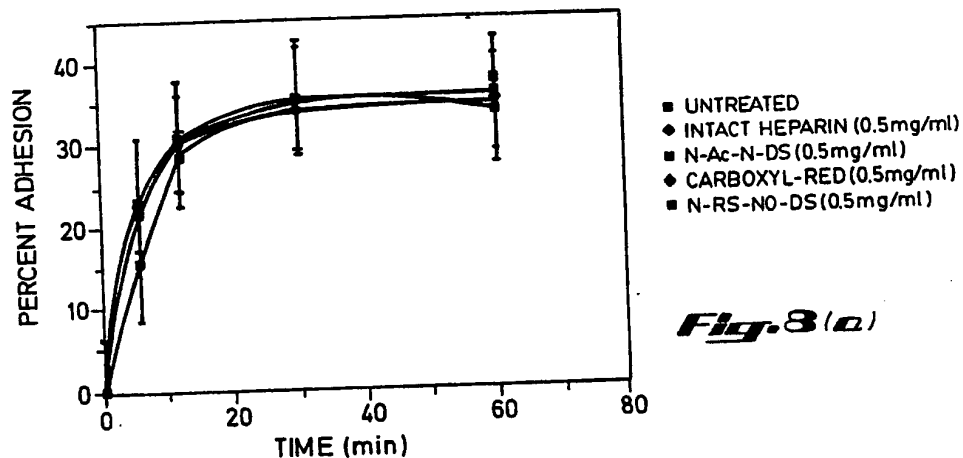
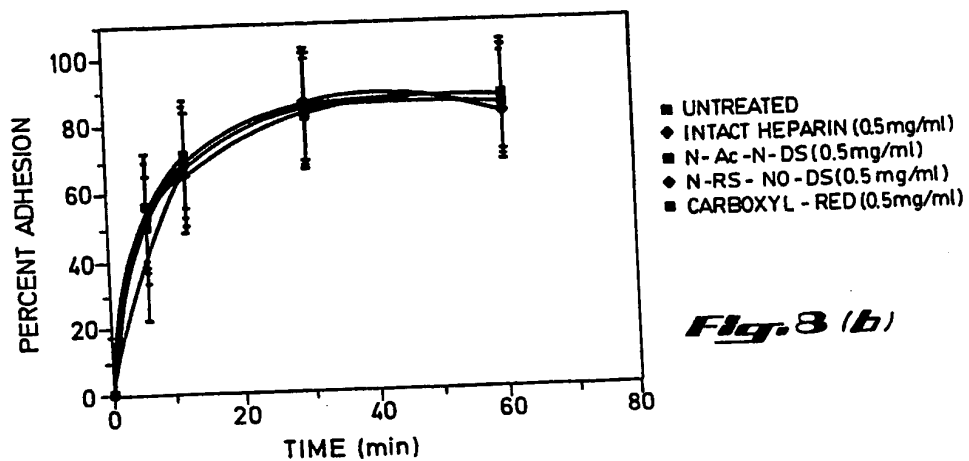
**Fig. 6**

EFFECTS OF MODIFIED HEPARINS ON THE GROWTH OF B16-BL6 MELANOMA CELLS

**Fig. 7**

SUBSTITUTE SHEET

5/6

EFFECTS OF MODIFIED HEPARIN-TREATMENT OF MELANOMA CELLS
ON THEIR ADHESION TO ENDOTHELIAL MONOLAYER*Fig. 8 (a)*ADHESION OF MELANOMA CELLS TO ENDOTHELIAL CELLS
PREVIOUSLY TREATED WITH MODIFIED HEPARINS*Fig. 8 (b)*

SUBSTITUTE SHEET

6/6

INHIBITION OF MELANOMA ADHESION TO LUNG TISSUE

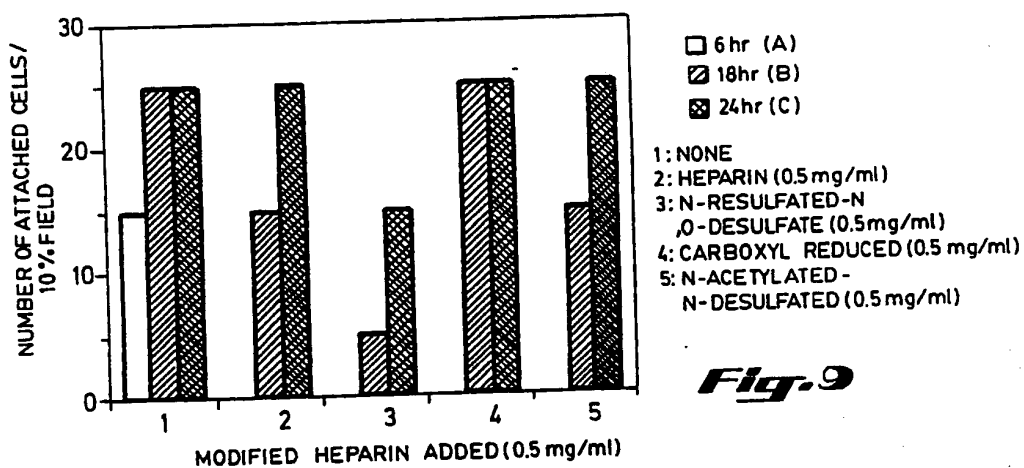


Fig. 9

INHIBITION OF MELANOMA INVASION TO LUNG

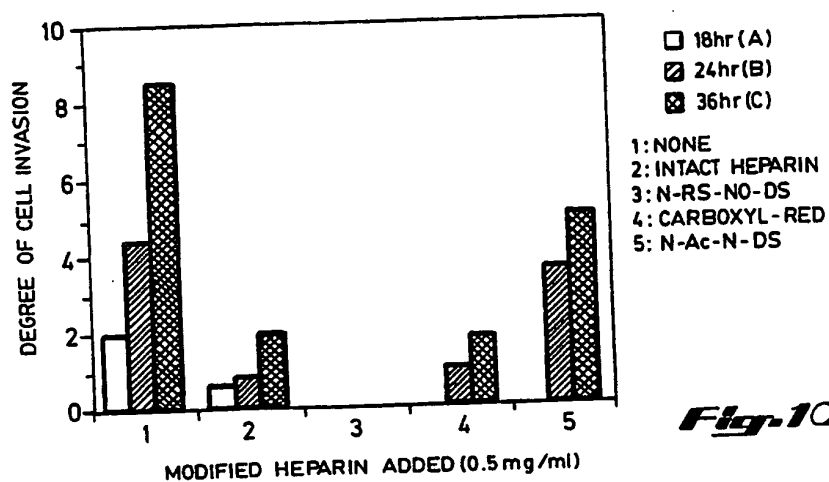



Fig. 10

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

PCT/US 91/04863

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C08B37/10; A61K31/725		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C08B ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,8 801 280 (BOARD OF REGENTS,THE UNIVERSITY OF TEXAS SYSTEM) 25 February 1988 THE WHOLE DOCUMENT ---	1-12
A	EP,A,0 176 769 (LANDSBERGER,ALBERT PROF.-DR.) 9 Apr11 1986 ---	
<p>¹⁰ Special categories of cited documents : ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
06 NOVEMBER 1991	22. 11. 91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	LENSEN H.W.M. 	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9104863
SA 49936**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/11/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8801280	25-02-88	AU-A- 7852687	08-03-88
EP-A-0176769	09-04-86	DE-A- 3432661	06-03-86
		US-A- 4738955	19-04-88

EPO FORM P0079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.